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13. Abstract (Maximum 200 Words) (abstract should contain no proprietary or confidential information) Our hypothesis is that inhibition of NFkB will be an effective treatment strategy for hormone refractory prostate cancer. We have identified that constitutive NFkB DNA in prostate cancer cell lines and Human Umbilical Venous Endothelial Cells (HUVECs). Moreover, we have found that parthenolide, is able to prevent NFkB DNA binding and inhibit <i>in vitro</i> cancer cell proliferation and angiogenesis as well as <i>in vivo</i> angiogenesis in a matrigel plug assay. Our work to date has shown that IRAK-1 and c-JUN are decreased in both hormone sensitive and hormone resistant cell lines by 6 hours of parthenolide treatment. This was done by use of a NFkB gene array. We have also shown that TRAF-1 is decreased by parthenolide in HUVECs. We have demonstrated that this corresponded to decreased NFkB DNA binding when whole cell extracts were exposed to parthenolide. The NFkB DNA binding experiments will be repeated using nuclear extracts. The parthenolide serum level achieved in mice that decreased <i>in vivo</i> angiogenesis was approximately 0.4 uM. In essence we have shown that genes change in response to parthenolide treatment and our efforts will focus on trying to confirm this at the protein level and determine whether other genes are affected at a lower concentration that may be independent of NFkB. This is due to the active <i>in vitro</i> level being 4 uM. Our first <i>in vivo</i> experiments suggested that parthenolide had no significant activity as a single agent, but as much activity as docetaxel. However, the combination of parthenolide and docetaxel had a pronounced effect. We need to perform further experiments to confirm this. Finally we have performed immunohistochemical staining of 97 prostate cancer specimens and noted that there is a graded increase in the presence of activated NFkB from normal prostate tissue to cancer specimens.			
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Introduction:

This grant has been assessing the following hypotheses:

- (1) Constitutive NFkB DNA binding present in hormone sensitive and hormone independent prostate cancer cell line can be inhibited by parthenolide and result in down regulation of proteins that drive the cancer process and angiogenesis.
- (2) Genes and proteins regulated by NFkB will be expressed at a greater level in hormone independent cell lines because of greater p65:p50 NFkB heterodimer DNA binding.
- (3) Parthenolide will augment the efficacy of growth factor deprivation, the anti-angiogenic effect of 2-methoxy-estradiol and the cytotoxic effects of docetaxel.
- (4) Parthenolide will be an effective agent to inhibit *in vivo* proliferation of prostate cancer cells.
- (5) Increased p65 subunit NFkB expression in prostatectomy specimens predicts for a poor clinical outcome.

The specific aims to address this hypothesis are:

- (1) Determine the genes that are transcribed by NFkB and drive the development of hormone independence and tumor associated angiogenesis
- (2) Identify the genes that are not transcribed due to the inhibition of parthenolide in prostate cancer cells and HUVECs.
- (3) Determine the ability of parthenolide to overcome hormone resistance, augment the activity of paclitaxel and another anti-angiogenic agent, 2-methoxyestradiol.
- (4) Examine the *in vivo* anti-neoplastic activity of parthenolide as a single agent and in combination with docetaxel and in another experiment in combination with 2-methoxyestradiol.
- (5) Determine the frequency and prognostic significance of NFkB activation in prostate cancer.

Body/Accomplishments/Outcomes

Specific Aims 1 and 2

Constitutive NFkB DNA binding present in hormone sensitive and hormone independent prostate cancer cell line can be inhibited by parthenolide and result in down regulation of proteins that drive the cancer process and angiogenesis.

Genes and proteins regulated by NFkB will be expressed at a greater level in hormone independent cell lines because of greater p65:p50 NFkB heterodimer DNA binding.

Prostate Cancer Cell Line Work:

In the grant proposal we had shown that parthenolide inhibited the growth of prostate cancer cells and inhibited NFkB DNA binding on gel shifts. We have commenced experiments evaluating the gene changes induced by parthenolide. Initially we proposed to perform ribonuclease protection assays. However, this very cumbersome process can be replaced and the evaluation be more easily accomplished by specific gene arrays. We have used this gene array technology with an array specific for NFkB from

“SuperArray”. Essentially, we have extracted total RNA from cells treated with and without parthenolide and observed for changes in genes under the control of NFkB. Figure 1a and 1b are examples of the results. We have observed the following changes qualitatively and plan to perform a more detailed analysis with software from “SuperArray” as well as confirm changes by analyzing protein with western blotting. The software will allow us to compare untreated hormone sensitive cell line (LNCaP) against its hormone resistant clone.

The most consistent and notable changes have been a decrease in IRAK-1 (interleukin-1 receptor-associated kinase) and c-JUN.

C4-2		LNCaP	
IRAK-1	c-JUN	IRAK-1	c-JUN

6 hours of parthenolide(10 uM)

Decrease	Decrease	Decrease	Decrease
----------	----------	----------	----------

24 hours of parthenolide

Decrease	Increase	Decrease	Increase
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Although this approach can be seen as a “fishing expedition” it has lead on us a direction that will further our understanding of parthenolide’s mechanism of action. We will confirm these changes by western blot analysis as well as evaluate for changes in phospho-c-JUN. This technology will also allow us to compare gene expression in the hormone sensitive compared with hormone resistant cell lines.

Angiogenesis Data

As detailed in the grant proposal we demonstrated the ability of parthenolide to prevent NFkB DNA binding, inhibit cell proliferation in human umbilical venous endothelial and capillary formation as well as inhibit angiogenesis *in vivo*. We also showed that bFGF and VEGF increased NFkB DNA binding. We developed an HPLC assay showing that the doses that inhibited angiogenesis *in vivo* were associated with detectable levels of parthenolide in the plasma (see table 1). However, the dose level achieved in the plasma was about one log lower than the dose that had an effect *in vitro*. This can be due to many reasons including uptake of parthenolide into the matrigel plug or repeated doses having an equivalent effect as a larger *in vitro* dose. We therefore plan to perform NFkB and apoptosis SuperArrays to determine what if any other genes may be effected at lower doses. In our follow-up *in vivo* experiments with cell lines injected in the flank we will also determine the concentration of the drug in the tumor mass after four weeks of treatment.

We have performed a ribonuclease protection assay (RPA) and found an increase TRAF-1 with VEGF and bFGF and that parthenolide decreased TRAF-1 in the presence and absence of these cytokines. We then confirmed this with RT-PCR.

Journal reviewers expressed concerns about two issues. The first is the issue of the EMSA (in original grant) was performed with whole cell lysates. Despite previous studies showing that the whole cell lysates mirrored the changes with nuclear extracts in previous experiments, we have opted to repeat the experiments with nuclear extracts. We will also perform this with the prostate cancer cell lines. There were also concerns about the quality of the pictures and the loading control of the RPA and RT-PCR (Figures 2 and

3). The RPA and RT-PCR are very complicated assays so we have decided to repeat the experiments using the NFkB superarray (seen above with C42 cells) and use western blot to confirm changes in the proteins. (Manuscript in preparation included)

Specific Aim 3

Parthenolide will augment the efficacy of growth factor deprivation, the anti-angiogenic effect of 2-methoxy-estradiol and the cytotoxic effects of docetaxel.

In 2002, the media provided by the company to support the growth of the 2C4 cells was defective and as a result our results were unreliable. We have rectified this problem and the cells are growing as expected and we plan to commence these experiments in the next month. We will be able to complete these experiments reliably in 2003. We also plan to perform similar analyses as well as nuclear extract NFkB DNA binding assays of the CWR22Rv1 cell line. This cell line is hormone independent and derived from a cell line that responds to antiandrogens (such as bicalutamide). This will allow us to use this therapy rather than the use of charcoal stripping to simulate the castrate state *in vitro* to see if we can make this cell line sensitive to hormonal therapy by pretreatment with parthenolide. If we define the presence of NFkB DNA binding, this cell line will be used in the *in vivo* experiments of specific Aim 4.

Specific Aim 4:

*Examine the *in vivo* anti-neoplastic activity of parthenolide as a single agent and in combination with docetaxel and in another experiment in combination with 2-methoxyestradiol.*

We have also completed our first *in vivo* experiment with C42 cells. These were implanted into the tibia of the mice. After six weeks, treatments were commenced and continued for 10 weeks. The results of the serum PSA test are seen in figure 4. We saw that parthenolide had no effect as a single agent but was not different from docetaxel. The combination of parthenolide plus docetaxel had a profound effect with no mice having PSA at the time of sacrifice. A similar finding has been seen by Dr Nakshatri who has been performing similar studies in breast cancer. However, the histopathological evaluation revealed low tumor take in the control group. Only one slide was examined per mouse and this finding may be due to a sampling error. We will examine all the slides to truly evaluate the tumor volume. This intraosseous orthotopic model is very difficult and if the tumor take is the issue we plan to repeat the experiments using a subcutaneous injection of either C42 or CWR22Rv1 in matrigel. The latter is a hormone resistant cell line that will also allow us to use an oral antiandrogen as a treatment so we can assess hormonal therapy (such as oral bicalutamide) with and without parthenolide to see if we can overcome hormone resistance with parthenolide. We have had success with this cell line in the laboratory with another experiment in the last 6 months and this type of approach was recently published in Clinical Cancer Research(1).

Figure 1a and Figure 1b: NF κ B Superarray
 - without parthenolide

- with 10 μ M parthenolide (6 hours)

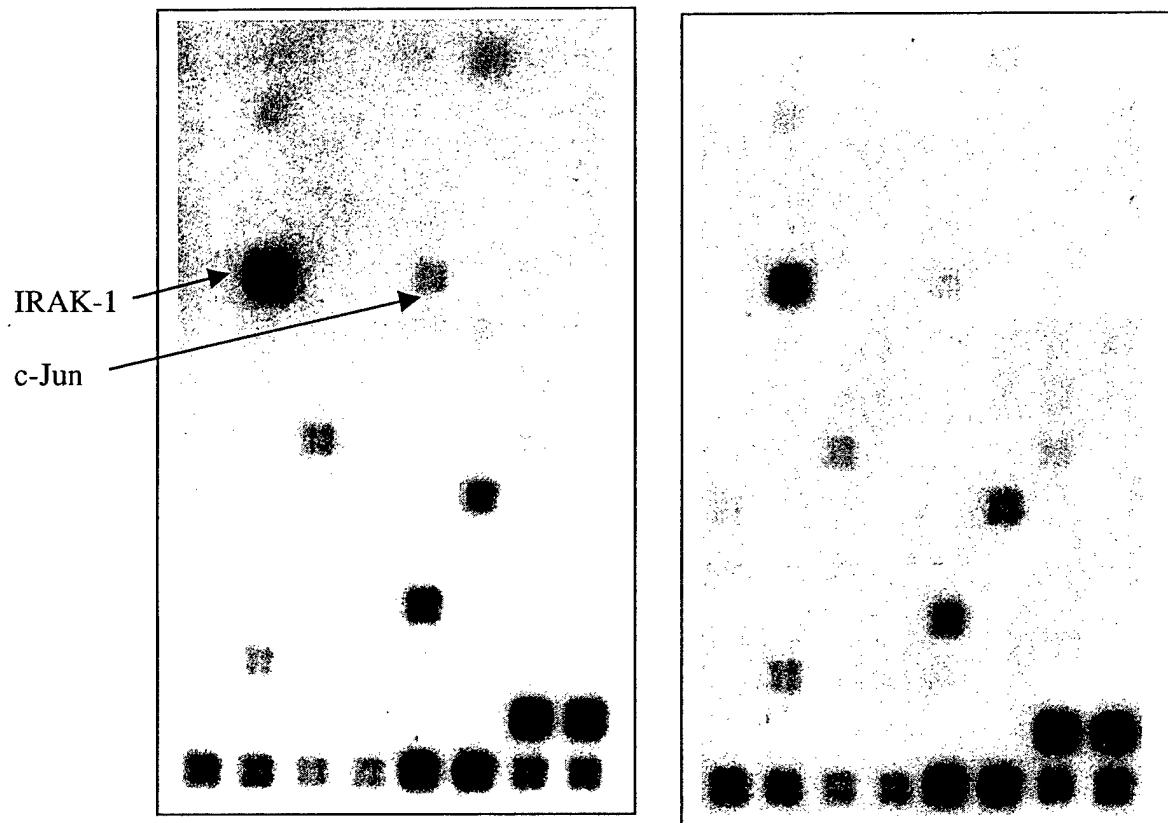


Table 1:

	VEGF OD - SE (%Inhibition)	bFGF OD - SE (%Inhibition)	Plasma Parthenolide concentration 1 hr after oral gavage
Control	4.55 - 0.37 100%	3.79 - 0.25 100%	0 μ M
Parthenolide 0.4 mg/kg	3.89 - 0.23 14% ($P=0.29$)	3.00 - 0.22 21% ($P=0.07$)	Undetectable
Parthenolide 4 mg/kg	4.01 - .27 12% ($P=0.46$)	2.26 - 0.24 41% ($P=0.0002$)	0.112 μ M
Parthenolide 40 mg/kg	3.60 - 0.26 21% ($P=0.09$)	2.08 - 0.30 45.3% ($P<0.0001$)	0.169 μ M

Figure 2

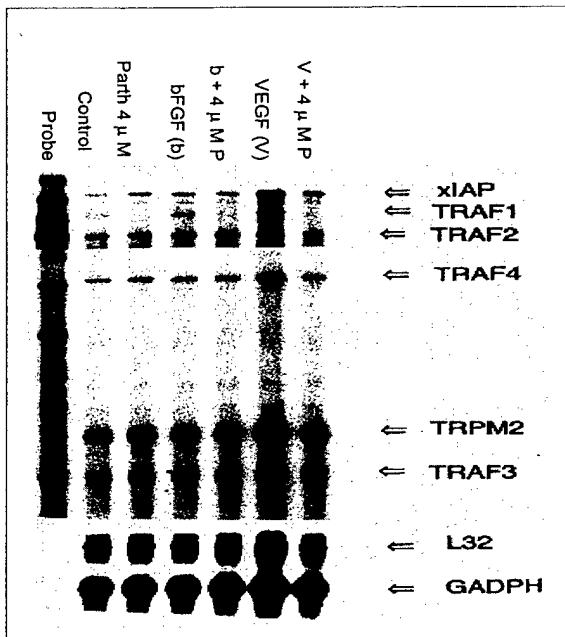


Figure 3

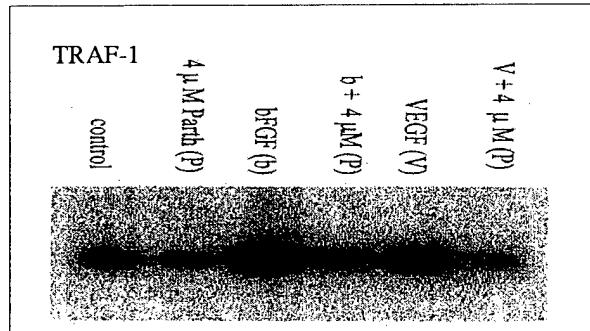
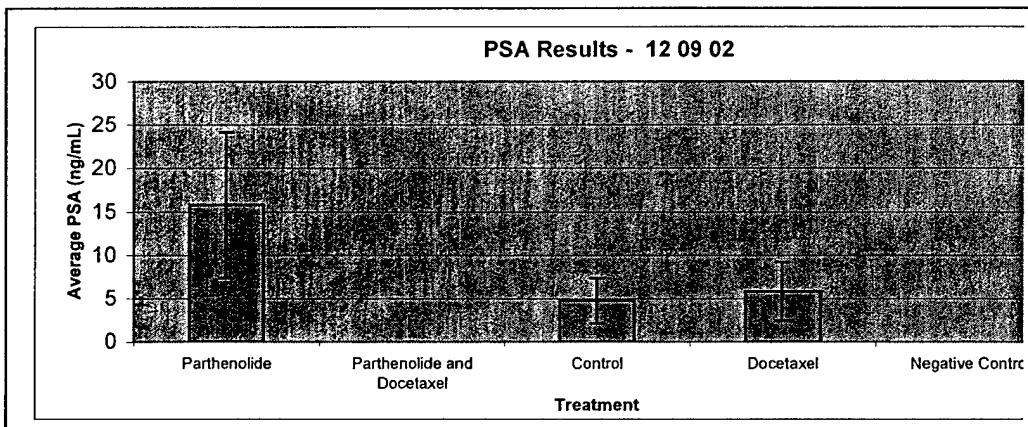


Figure 4:



Treatment	N	Average PSA (ng/mL)	SE	number with high PSA
Parthenolide	13	15.764	8.395	3
Parthenolide and Docetaxel	12	0	0	0
Control	16	4.756	2.595	4
Docetaxel	13	5.805	3.381	3

Specific Aim 5:

Determine the frequency and prognostic significance of NF κ B activation in prostate cancer

Clinicopathological Experiments:

Ninety seven cases of radical prostatectomy were analyzed by immunohistochemistry. The antibody employed was a rabbit polyclonal antibody that identifies the binding site of the p65 subunit of the Rel family. (Santa Cruz Biotechnology, Santa Cruz, CA). Serial 5- μ m-thick sections of formalin-fixed slices of radical prostatectomy specimens were used for the studies. The tissue blocks containing the highest Gleason score and the maximum amount of tumor were selected. Immunoreactivity for the p65 subunit was seen in 90%, 99% and 100% of all the specimens. However, the amount of immunoreactivity was greater in the neoplastic lesions compared with the benign glands. Specifically, the mean percent staining intensity was 14.7% in the benign glands and 79.2% in PIN and 88.7% in the adenocarcinoma. The difference in amount of staining was significantly greater in the PIN lesions compared with the benign glands ($p < 0.001$) and was greater in the invasive neoplastic disease compared with the intraepithelial neoplasia ($p < 0.001$)

Staining	Proportion p65 Present	Mean Staining (SD)	Range
Normal	90%	14.7% (17.1)	0-50
PIN	99%	79.2 (17.8)	0-95
Cancer	100%	88.7% (12.1)	35-95

Intensity	0	1	2	3
Normal	9(9%)	57(58%)	31(32%)	0(0%)
PIN	1(1%)	10(10%)	62(64%)	24(24%)
Cancer	0(0%)	0(0%)	29(29%)	68(70%)

The differential staining (frequency and amount) and graded increase between the normal, PIN and cancerous areas supports the contention that staining for the I κ B binding site on the p65 subunit does represent activated NF κ B. The presence of staining in all the cancer specimens support the *in vitro* data that activated NF κ B is a common finding. The low amount of nuclear staining is probably due to the very short half life of transcription factors in the nucleus. The lack of association between pathological and clinical prognostic features is secondary to the universal staining at either 2+ or 3+ intensity.

(Manuscript in preparation)

Conclusions:

We have shown that NFkB is present in prostate cancer cells *in vitro* and in all clinical specimens of prostate cancer. This supports the notion that this is a relevant transcription factor to target for the treatment of prostate cancer. We have verified that parthenolide can inhibit NFkB *in vitro* and is orally bioavailable and able to inhibit angiogenesis *in vivo* and possibly augment the efficacy of docetaxel. We have also demonstrated that parthenolide can inhibit genes under control of NFkB (IRAK-1, c-JUN, TRAF-1). Our goals in the next 12 months are to complete the experiments required to confirm these findings. Specifically evaluate for protein changes after exposure to parthenolide and use the "SuperArray" software to more precisely compare the genes under NFkB control that are expressed in treated compared with untreated cells and hormone sensitive compared with hormone resistant cell lines. Our initial *in vivo* experiments have demonstrated an ability to augment the efficacy of docetaxel and we will perform follow-up studies. These questions will also be evaluated *in vitro*. We are also preparing to determine whether we can restore hormone sensitivity with parthenolide *in vitro* and *in vivo* using the CWR22Rv1 cell line. This may occur if parthenolide is able to alter NFkB genes driving hormone resistance. Gene array experiments of the CWR22Rv1 cell line with and without parthenolide will help us evaluate for possible biochemical explanations if this is observed.

The other question to be addressed is why did parthenolide have an *in vivo* effect at 0.4 uM whereas the active *in vitro* dose was less about 4uM. This can be due to many reasons including uptake of parthenolide into the cells or repeated doses having an equivalent effect as a larger *in vitro* dose. We therefore plan to perform NFkB and apoptosis SuperArrays to determine what if any other genes may be effected at the lower 0.4uM dose and compare with the 4uM dose. In our follow-up *in vivo* experiments with cell lines injected in the flank we will also determine the concentration of the drug in the tumor mass after four weeks of treatment.

References:

1. Sirotnak, F. M., She, Y., Lee, F., Chen, J., and Scher, H. I. Studies with CWR22 xenografts in nude mice suggest that ZD1839 may have a role in the treatment of both androgen-dependent and androgen-independent human prostate cancer. *Clin Cancer Res*, 8: 3870-3876, 2002.

INHIBITION OF ANGIOGENESIS BY PARTHENOLIDE: A NUCLEAR
FACTOR KAPPA B INHIBITOR DERIVED FROM *TENACETUM*
PARTHENEUM

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ABSTRACT

Purpose: The transcription factor, Nuclear Factor kappa B (NFkB), is a “master-switch” that activates a variety of genes including some associated with angiogenesis. In this paper we evaluated the ability of parthenolide, a NFkB-DNA inhibitor, to impact upon this process.

Experimental Design: The presence of NFkB DNA binding in HUVECs was evaluated by electromobility gel shift assay. Genes under the control of NFkB DNA binding and inhibited by parthenolide were evaluated by ribonuclease protection assay. The notable finding from this experiment was confirmed by southern blotting of products obtained from reverse transcription-polymerase chain reaction. Functional effects of parthenolide upon HUVECs were evaluated by assays of proliferation, capillary formation and apoptosis. *In vivo* activity was verified by a matrigel plug assay in nude mice and a high-pressure liquid chromatography assay was employed to determine the serum concentration of parthenolide one hour after oral gavage.

Results: NFkB DNA binding was present in HUVECs and (i) increased by bFGF and VEGF and (ii) inhibited by parthenolide at 4 μ M. These findings were associated with increased binding and subsequent inhibition of a gene under NFkB control - Tumor Necrosis Factor Receptor Associated Factor (TRAF-1). Parthenolide also inhibited endothelial cell proliferation and capillary formation and induced apoptosis. Oral administration was also able to abrogate *in vivo* angiogenesis and provide detectable plasma concentrations of parthenolide.

Conclusion: Parthenolide inhibits NFkB DNA binding in HUVECs and at least one gene under its control and this is associated with anti-angiogenic effects both *in vitro* and *in-vivo*.

INTRODUCTION

Nuclear Factor-kappa B (NFkB) is a transcription factor that controls many cell functions. It is sequestered in the cytoplasm bound to the inhibitory protein, I-kappa B (IkB). Extracellular signals activate NFkB by releasing it from IkB. These signals emanate from stimuli including cytokines such as Tumor Necrosis Factor (TNF) and interleukin-1 (IL-1), viral infections, phorbol esters, oxidants, chemotherapy and radiation(1). Once activated NFkB binds to DNA and results in transcription of many genes including those involved in (1) invasion – interleukin 6 (IL-6), urokinase plasminogen activator (uPA), matrix metallaproteinase 9; (2) new blood vessel development (angiogenesis) – interleukin 8 (IL-8), vascular endothelial growth factor (VEGF); (3) inhibition of programmed cell death (apoptosis) – Inhibitors of apoptosis genes (IAP), c-IAP 1, c-IAP 2, TNF receptor-associated factors - TRAF-1 and TRAF-2, Bfl-1/A1, Bcl-X_L and manganese superoxide dismutase (Mn-SOD); and (4) inflammation – cyclo-oxygenase-2(2-4)

Activation of NFkB has been shown to cause TNF and chemotherapy resistance and to induce genes (TRAF-1, TRAF-2, c-IAP-1, and c-IAP-2) that inhibit TNF and chemotherapy induced apoptosis(5). In contrast to the usual control mechanisms of non-cancerous cells, NFkB has been found to be constitutively activated in many malignancies including lymphoma, leukemia, melanoma, squamous cell carcinoma, ovarian, breast and pancreatic cancer cell lines(6-8). Inhibition of NFkB activation by a sesquiterpene lactone, parthenolide has been shown to decrease *in vitro* cancer cell growth and enhance taxane induced cytotoxicity(9). Parthenolide is thought to be the active ingredient in feverfew, a herbal remedy that is used to prevent migraines and treat

arthritis(10). Feverfew is derived from the plant *Tanacetum parthenium*. Parthenolide inhibition of cellular proliferation is associated with reduced NFkB binding to DNA and decreased expression of the anti-apoptotic genes under NFkB control. Parthenolide inhibits NFkB by stabilizing the binding of NFkB to its inhibitor, I_kB which is thought to be mediated by inhibiting the I- κ B kinase complex(11, 12). Recently, inhibition of NFkB by insertion of mutated I_kB into cancer cell lines has been shown to result in decreased vascular endothelial growth factor (VEGF) and IL-8 expression. This resulted in decreased *in vivo* growth with decreased angiogenesis of an ovarian cancer cell and melanoma cell line(13, 14). We therefore set out to explore the anti-angiogenic properties of parthenolide.

MATERIALS AND METHODS

Materials: Human umbilical venous endothelial cells (HUVECs) (Clonetics, San Diego CA) were cultured in EBM-2 media (Clonetics, San Diego CA) and harvested after having undergone no more than five passages. Parthenolide in 100% alcohol was added at varying concentrations to a proliferation and a capillary formation assay. VEGF (Chemicon International, Temecula, CA) and basic Fibroblast Growth Factor, bFGF (R&D Systems Minneapolis, MN) were added to the assays in doses that have been shown *in vitro* to induce the maximal amount of HUVEC endothelial cell proliferation(15). Thrombin, bovine fibrinogen and aprotinin, (all from Sigma Chemical Co, St Louis MO) were used for the formation of a fibrin clot. Microcarrier beads consisting of thin layer denatured collagen chemically coupled to a matrix of cross-linked dextran (175 microns, Cytodex™3, Amersham Pharmacia, Biotech AB, Uppsala Sweden) were employed as the base for the capillary formation. This assay has been shown to induce capillaries with identifiable lumens(16). The MTS/PMS system (Promega, Madison WI) was used to assess cell viability for the proliferation assay.

Cells and Treatment

Human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego, CA) were grown in EBM-2 media (Clonetics, San Diego, CA) until 80% were confluent. Experiments were done with and without supplements. HUVEC media (HM) refers to media plus all the components of “EGM-2 SingleQuot” added to a 500 mL bottle of media unless otherwise stated. Components in one “EGM-2 SingleQuot” include: epidermal growth factor (0.5 ml), hydrocortisone (0.2 ml), fetal bovine serum (25 mL),

vascular endothelial growth factor (0.5 mL), basic fibroblast growth factor (2 mL), recombinant IGF-1 (0.5 mL), ascorbic acid (0.5 mL), GA-1000 (0.5 mL) and heparin. Media with no supplements refers to base media only. Cells were exposed to additional basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN) and vascular endothelial cell growth factor (VEGF) (Chemicon International, Temecula, CA) as detailed below. Drugs were added as described below.

Electromobility Membrane Supershift Assay

HUVECS were plated on 100 mm plates and harvested in exponential growth phase. Drugs, antibodies and cytokines were added three hours prior to harvesting – bFGF alone at 50 ng/mL, VEGF alone at 100 ng/mL, and parthenolide at doses ranging of 0.5 and 4 μ M. Whole cell extracts were made and incubated with a radiolabelled NFkB probe for 30 minutes at room temperature. The protein probe binds to the NFkB DNA binding site in the promoter region of the immunoglobulin gene. Electrophoresis and autoradiography were performed as described previously(7) using NF-kB and SP-1 probes (Promega, Madison, WI). The specificity of the drug inhibiting NFkB DNA binding was verified by the use of the SP-1 probe as a control. STAT-3 and STAT-5 probes were also employed (Santa Cruz Biotechnology, Santa Cruz, CA).

Ribonuclease Protection Assay

Ribonuclease protection assay was performed on mRNA extracted from the HUVECs using Trizol reagent (Life Technologies, Inc., Rockville, MD) according to the

manufacturer's instructions. The HUVECs were treated with solvent control and with parthenolide (4 μ M) for 2 hours to determine the molecular changes resulting from NFkB inhibition in these cell lines. Multi-probe templates (hAPO-5 and hAPO-2C - PharmingenTM) containing DNA of relevance for NFkB were used(9). Total RNA from HUVECs was isolated. Ten μ g of RNA was subjected to RNase protection using the hAPO-5 and h APO-2C probes (Pharmingen, San Diego, CA) as described by the manufacturers.

Reverse Transcription-PCR (RT-PCR) and Southern blotting to confirm TRAF1 expression

Total RNA from HUVECs was prepared by Trizol reagent (Life Technologies, Inc., Rockville, MD) method according to the manufacturer's instructions. 100 ng of RNA was reverse-transcribed by using Superscript one-step RT-PCR with Platinum Taq kit (Life Technologies, Inc., Rockville, MD) as recommended by the manufacturers. As a control, mRNA corresponding to the ribosomal protein gene 36B4 was also amplified. The optimized linear range of amplification was determined as being 30 cycles for TRAF1 and 20 cycles for 36B4. Primers used for TRAF1 and 36B4 are as follows:

- a) TRAF1, 5' -TCACCTCCCAGACCTCCAC-3' (forward primer) and 5'-ACGCAGCTTCCCCTCCAGCT-3' (reverse primer). b) 36B4, 5'-TGGAGAAACTGCTGCCTCAT-3' (forward primer) and 5'-GGAGATGTTGAGCATGTTCA-3' (reverse primer). PCR products were visualized by Southern blotting(17).

Proliferation: HUVECs were plated in a 96-well U-bottomed plate (Becton Dickinson Labware, Franklin Lakes, NJ) at a concentration of 10,000 cells per 50 microliters (μ L) of media and incubated in 5% CO₂ at 37°C for 48 hours. Varying drug concentrations in 50 μ L of media were added to the media and this mixture was added to each well within one hour of the HUVECs being seeded. The proliferation experiments were performed with and without stimulation by the addition of VEGF (60 ng/mL) and bFGF (20 ng/mL). These factors in high doses were chosen to partially simulate the tumor microenvironment. Colorimetric readings were obtained using the MTS/PMS system and an ELISA plate reader. The readings obtained for each concentration tested were from an average of eight wells. Each experiment was expressed as a percentage of the solvent control and completed at least three times with consistent results. The results presented are an average of at least 3 experiments.

Endothelial cell capillary formation: Two hundred milligrams of microcarrier beads suspended in PBS were autoclaved and then added to HUVECs at a concentration of 30 HUVECs per microcarrier bead. Microcarrier beads and cells were added to a siliconized petri dish and rocked at 37°C in 5% CO₂ for 48 hours. The HUVEC coated microcarrier beads were transferred to a fibrin clot solution. Fibrinogen was dissolved at 2.5 mg/mL in PBS with 0.15 U/mL of aprotinin. Approximately 20 HUVEC coated microcarrier beads were added to each well of a 12 well plate and then thrombin (0.625 U/mL) was added to form a gelatinous clot. Media (1.5 mLs) with 1% human serum and aprotinin (0.15 U/mL) were added to the top of each clot. The addition of VEGF(60 ng/mL) and bFGF (20 ng/mL) was required to ensure robust capillary formation. There was minimal

capillary formation without stimulation and therefore all results reported are with stimulation. Parthenolide was added to the top layer. Capillary formation was then quantified after four days: every capillary greater than the radius of the bead was scored and the average number of tubules for each bead per well was determined. The results were expressed as a fraction of the positive control. The experiments were repeated at least three times and the results presented are the average of at least three experiments.

Measurement of Apoptosis

Apoptosis was measured using the Cell Death Kit (Roche). HUVECs were plated in 96 - well U-bottomed plate and parthenolide was added 3 hours after plating. Cell lysates were prepared after 24 hours and placed into a streptavidin-coated microplate. A mixture of anti-histone- biotin and anti-DNA-POD was added and incubated for 2 hours. The presence of apoptosis was identified by the anti-histone antibody binding to the histone-component of the nucleosomes which simultaneously fixes the immunocomplex to the streptavidin-coated microplate via its biotinylation. Additionally, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. After removal of unbound antibodies by a washing step, the amount of nucleosomes is quantified by the POD retained in the immunocomplex. POD is determined photometrically with ABTS (2,2'-Azino-di[3-ethyl-benz-thiazolin- sulfonate]) as substrate.

In vivo Analysis of Angiogenesis by Matrigel:

Low growth factor "Matrigel" (Becton Dickinson, Bedford, MA) was supplemented with 200 ng/mL of VEGF in the left flank and 50 ng/mL of bFGF in the right flank. Treatment was commenced the day after injection of the Matrigel. Treatment groups consisted of

15 mice per cohort and were treated with either solvent control, 0.4 mg/kg, 4 mg/kg or 40 mg/kg on days. After 14 days of treatment each plug was resected, weighed and immersed in 10 mL of distilled water per gram of Matrigel plug. The mixture was placed in a shaking water bath for 24 hours. After 24 hours, 100 microliters was mixed with 100 microliters of Drabkin's reagent (Sigma Chemical Co, St Louis MO) for 15 minutes at room temperature and then placed in a 96 well plate. The hemoglobin concentration was determined by absorbance readings on a plate reader and reported as an optic density (OD). Two OD readings were recorded per sample with the average reported for each. The result for each treatment group was reported as the average from the fifteen mice in each cohort. The presence of statistical differences between control and treatment was evaluated by the 2-sample 2-sided T-test without log transformation.

High Pressure Liquid Chromatography Assay

High pressure liquid chromatography assay with mass spectroscopy (HPLC-MS) was used to determine the plasma parthenolide concentration in mice one hour after oral gavage. Briefly, a Shimadzu 8000 alpha HPLC-MS system was employed. The mobile phase consisted of 7% acetonitrile, 63% methanol, and 30% water acidified to pH 2.2 with trifluoroacetic acid (0.05%) delivered isocratically at a flow of 0.2 ml/min. The column used was a Phenomenex Synergi Polar RP (4 μ particle size, 250 cm x 2 mm) with a guard column. The sample is ionized using an APCI probe in the positive mode. The mass spectrometer conditions were: probe temperature 325 $^{\circ}$ C, CDL temperature 190 $^{\circ}$ C, detector voltage 1.7 volts, and nitrogen flow of 2.5 L/min. The internal standard used for quantifying the amount of parthenolide was hesperatin.

RESULTS

Molecular Effects of Nuclear Factor kappa B Inhibition by Parthenolide in Human Umbilical Venous Endothelial Cells (HUVECs)

When the HUVECS were analyzed by electromobility shift assay NFkB DNA binding was observed (Figure 1a). In HUVEC media without supplements (panels 1-3) constitutive NFkB DNA binding was noted to be slightly increased in the presence of excess bFGF (50 ng/mL) and VEGF (100 ng/mL). It is of note that NFkB DNA binding was decreased in presence of supplements and this can be attributed to the hydrocortisone or possibly other products in fetal bovine serum. However, parthenolide was able to decrease this NFkB DNA binding further at 4 μ M. This experiment also shows increase NFkB DNA binding with 50 ng/mL of bFGF and 100 ng/mL of VEGF (compare panels 7 and 4; 10 and 4 respectively). Even in the presence of excess cytokines (bFGF and VEGF), parthenolide was able to inhibit NFkB in the low micromolar range.

The specificity of parthenolide for inhibiting NFkB DNA binding in endothelial cells was determined by the fact that it did not inhibit SP-1, STAT-5, nor STAT-3 binding (Figure 1 b).

To define the anti-apoptotic genes activated by NFkB in HUVECs we employed a ribonuclease protection assay. We observed a basal level of TRAF-1 in HUVECS that was decreased by parthenolide (Figure 2a). Moreover, both bFGF and VEGF increased the amount of TRAF-1 mRNA which was also blocked by parthenolide. This effect was confirmed by Reverse-Transcriptase Polymerase Chain Reaction and Southern blotting

(Figure 2b). Other anti-apoptotic genes under NFkB control, TRAF-2, TRAF-4, TRPM-2, TRAF-3 were not altered by the cytokines or parthenolide. It is also worth noting that these cells do not express other major NFkB inducible anti-apoptotic genes, c-IAP-1 or c-IAP-2 (data not shown). These cells also expressed bclw, bclx, bad, bak and bcl2 (data not shown). The expression of these, most notably bclx, were not altered by parthenolide.

Phenotypic Effects of Nuclear Factor kappa B Inhibition by Parthenolide in HUVECs

Inhibition of NFkB resulted in decreased HUVEC proliferation in a dose dependent manner between 5 μ M and 10 μ M with 50% inhibition (IC₅₀) at approximately 7.5 μ M (Figure 3a). Exposure to the survival factors, VEGF and bFGF, were slightly able to protect the HUVECs from parthenolide. The more phenotypically complex function of forming capillaries was inhibited at lower concentrations with an IC₅₀ of 3.2 μ M (Figure 3b). We then confirmed that parthenolide was cytotoxic by finding that parthenolide induced apoptosis in a dose dependent manner that correlated with the proliferation assay findings. Namely, apoptosis was observed at 5 μ M but not below and a greater amount was seen at 10 μ M (Figure 3c).

To determine whether this finding was of biological relevance we performed an *in vivo* assay of angiogenesis with Matrigel plugs. In these *in vivo* experiments, the mice were treated by daily oral gavage for 2 weeks with varying doses of parthenolide. There was a dose response inhibition of bFGF induced angiogenesis (Table 1): 21% inhibition with 0.4 mg/kg (not statistically significant) but 41% inhibition by 4 mg/kg and 45% with 40 mg/kg ($P < 0.001$). In contrast there was only 15 to 20% inhibition of VEGF induced

angiogenesis by all doses of parthenolide (not statistically significant). To determine the plasma concentrations required for a biological effect we employed high-pressure liquid chromatography. No detectable drug concentrations were noted at one hour in the 0.4 mg/kg cohort which had no biological effect. In contrast, parthenolide concentrations were detectable at the doses that did have an *in vivo* biological effect. Namely, 4 mg/kg and 40 mg/kg doses resulted in concentrations of 28.11 ng/mL (0.112 μ M) and 42.21 ng/mL (0.169 μ M) respectively.

DISCUSSION:

Agents that specifically attack targets that drive medical diseases are coming to the forefront. They are needed to advance the treatment of many disorders including cancers and inflammatory disorders. In this paper we have shown that parthenolide inhibits NFkB DNA binding in HUVECs and that this inhibition was associated with inhibition of TRAF-1, an anti-apoptotic gene under its control. This gene has been shown to be important in preventing TNF and chemotherapy induced apoptosis(5). It was also detailed that parthenolide inhibited HUVEC proliferation, capillary formation and induced apoptosis. Previous investigators have shown that treatment with antisense NF-kappaB oligonucleotide completely blocked IL-8 induced tubular morphogenesis(18). All this data would suggest that the *in vitro* effects are at least in part due to inhibition of NFkB DNA binding in endothelial cells. The induction apoptosis is also consistent with the previous reports that inhibition of NFkB DNA binding was associated with induction of polyADP ribosyltransferase (PARP)-degradative apoptosis in breast cancer and leukemia cells lines(9, 19).

Parthenolide has been shown to inhibit a member of the signal transducers and activators of transcription family (STAT 3) which has been shown to be important in carcinogenesis and is upregulated by IL-6(20). In HUVECs however, neither STAT-3 nor STAT-5 were effected by parthenolide. Interestingly, STAT-5 is regulated by NFkB in the developing mammary gland(21). Inhibition of NFkB with parthenolide however did not affect STAT-5 binding in HUVECs. These angiogenic effects suggest the effects of parthenolide were not mediated by inhibition of either STAT pathway.

VEGF and bFGF are important cytokines in angiogenesis and have clearly been shown to be survival factors for endothelial cells(22-24). The interaction between VEGF and NFkB has been reported previously. It has been shown that that VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin mRNAs was mainly through NF-kappaB activation(25) Here we have demonstrated that both bFGF and VEGF increase NFkB DNA binding and transcription of TRAF-1. Although the association between NFkB and TRAF-1 is well characterized, this data is the first to show a correlation of TRAF-1 in endothelial cells and a possible relationship with angiogenesis(26). It is also worth noting that these cytokines partially protected the endothelial cells from parthenolide as there was a modest increase in the parthenolide dose needed to inhibit endothelial cell proliferation.

Finally, we have shown that parthenolide is worthy of clinical evaluation as it was able to suppress bFGF induced angiogenesis *in vivo* to a significant degree. Detectable plasma concentrations of parthenolide in the mice were correlated with this pharmacodynamic outcome. Preliminary pharmacokinetic evaluations detail the presence of a correlation between detectable plasma concentrations of parthenolide and inhibition of angiogenesis. The number of plasma samples analyzed limits the comments that can be made concerning the disparity between the effective *in vitro* and *in vivo* doses. Furthermore, it supports the data that parthenolide may be a viable product for the treatment of patients with diseases mediated by NFkB and/or NFkB mediated angiogenesis such as cancer and inflammatory disorders such as psoriasis and arthritis.

REFERENCES

1. Baeuerle, P. A. and Baltimore, D. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell*, *53*: 211-217, 1988.
2. Grumont, R. J., Rourke, I. J., and Gerondakis, S. Rel-dependent induction of A1 transcription is required to protect B cells from antigen receptor ligation-induced apoptosis. *Genes Dev*, *13*: 400-411, 1999.
3. Jones, P. L., Ping, D., and Boss, J. M. Tumor necrosis factor alpha and interleukin-1beta regulate the murine manganese superoxide dismutase gene through a complex intronic enhancer involving C/EBP-beta and NF-kappaB. *Mol Cell Biol*, *17*: 6970-6981, 1997.
4. Zong, W. X., Edelstein, L. C., Chen, C., Bash, J., and Gelinas, C. The prosurvival Bcl-2 homolog Bfl-1/A1 is a direct transcriptional target of NF-kappaB that blocks TNFalpha-induced apoptosis. *Genes Dev*, *13*: 382-387., 1999.
5. Wang, C. Y., Mayo, M. W., Korneluk, R. G., Goeddel, D. V., and Baldwin, A. S., Jr. NF-kappaB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. *Science*, *281*: 1680-1683., 1998.
6. Giri, D. K. and Aggarwal, B. B. Constitutive activation of NF-kappaB causes resistance to apoptosis in human cutaneous T cell lymphoma HuT-78 cells. Autocrine role of tumor necrosis factor and reactive oxygen intermediates. *J Biol Chem*, *273*: 14008-14014, 1998.

7. Nakshatri, H., Bhat-Nakshatri, P., Martin, D. A., Goulet, R. J., Jr., and Sledge, G. W., Jr. Constitutive activation of NF-kappaB during progression of breast cancer to hormone-independent growth. *Mol Cell Biol*, 17: 3629-3639., 1997.
8. Wang, W., Abbruzzese, J. L., Evans, D. B., Larry, L., Cleary, K. R., and Chiao, P. J. The nuclear factor-kappa B RelA transcription factor is constitutively activated in human pancreatic adenocarcinoma cells. *Clin Cancer Res*, 5: 119-127., 1999.
9. Patel, N. M., Nozaki, S., Shortle, N. H., Bhat-Nakshatri, P., Newton, T. R., Rice, S., Gelfanov, V., Boswell, S. H., Goulet, R. J., Jr., Sledge, G. W., Jr., and Nakshatri, H. Paclitaxel sensitivity of breast cancer cells with constitutively active NF-kappaB is enhanced by IkappaBalphalpha super-repressor and parthenolide. *Oncogene*, 19: 4159-4169., 2000.
10. Johnson, E. S., Kadam, N. P., Hylands, D. M., and Hylands, P. J. Efficacy of feverfew as prophylactic treatment of migraine. *Br Med J (Clin Res Ed)*, 291: 569-573, 1985.
11. Hehner, S. P., Heinrich, M., Bork, P. M., Vogt, M., Ratter, F., Lehmann, V., Schulze-Osthoff, K., Droege, W., and Schmitz, M. L. Sesquiterpene lactones specifically inhibit activation of NF-kappa B by preventing the degradation of I kappa B-alpha and I kappa B-beta. *J Biol Chem*, 273: 1288-1297, 1998.
12. Bork, P. M., Schmitz, M. L., Kuhnt, M., Escher, C., and Heinrich, M. Sesquiterpene lactone containing Mexican Indian medicinal plants and pure sesquiterpene lactones as potent inhibitors of transcription factor NF-kappaB. *FEBS Lett*, 402: 85-90, 1997.

13. Huang, S., Robinson, J. B., Deguzman, A., Bucana, C. D., and Fidler, I. J. Blockade of nuclear factor-kappaB signaling inhibits angiogenesis and tumorigenicity of human ovarian cancer cells by suppressing expression of vascular endothelial growth factor and interleukin 8 [In Process Citation]. *Cancer Res*, *60*: 5334-5339, 2000.
14. Huang, S., DeGuzman, A., Bucana, C. D., and Fidler, I. J. Nuclear factor-kappaB activity correlates with growth, angiogenesis, and metastasis of human melanoma cells in nude mice [In Process Citation]. *Clin Cancer Res*, *6*: 2573-2581, 2000.
15. Yoshida, S., Ono, M., Shono, T., Izumi, H., Ishibashi, T., Suzuki, H., and Kuwano, M. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol Cell Biol*, *17*: 4015-4023, 1997.
16. Nehls, V. and Drenckhahn, D. A novel, microcarrier-based in vitro assay for rapid and reliable quantification of three-dimensional cell migration and angiogenesis. *Microvasc Res*, *50*: 311-322, 1995.
17. Bhat-Nakshatri, P., Newton, T. R., Goulet, R., Jr., and Nakshatri, H. NF-kappaB activation and interleukin 6 production in fibroblasts by estrogen receptor-negative breast cancer cell-derived interleukin 1alpha. *Proc Natl Acad Sci U S A*, *95*: 6971-6976, 1998.
18. Shono, T., Ono, M., Izumi, H., Jimi, S. I., Matsushima, K., Okamoto, T., Kohno, K., and Kuwano, M. Involvement of the transcription factor NF-kappaB in tubular morphogenesis of human microvascular endothelial cells by oxidative stress. *Mol Cell Biol*, *16*: 4231-4239, 1996.

19. Gelfanov, V. M., Burgess, G. S., Litz-Jackson, S., King, A. J., Marshall, M. S., Nakshatri, H., and Boswell, H. S. Transformation of interleukin-3-dependent cells without participation of Stat5/bcl-xL: cooperation of akt with raf/erk leads to p65 nuclear factor kappaB-mediated antiapoptosis involving c-IAP2. *Blood*, **98**: 2508-2517, 2001.
20. Sobota, R., Szwed, M., Kasza, A., Bugno, M., and Kordula, T. Parthenolide inhibits activation of signal transducers and activators of transcription (STATs) induced by cytokines of the IL-6 family. *Biochem Biophys Res Commun*, **267**: 329-333, 2000.
21. Geymayer, S. and Doppler, W. Activation of NF-kappaB p50/p65 is regulated in the developing mammary gland and inhibits STAT5-mediated beta-casein gene expression. *Faseb J*, **14**: 1159-1170, 2000.
22. Ferrara, N. The role of vascular endothelial growth factor in pathological angiogenesis. *Breast Cancer Res Treat*, **36**: 127-137, 1995.
23. Dinney, C. P., Bielenberg, D. R., Perrotte, P., Reich, R., Eve, B. Y., Bucana, C. D., and Fidler, I. J. Inhibition of basic fibroblast growth factor expression, angiogenesis, and growth of human bladder carcinoma in mice by systemic interferon- alpha administration. *Cancer Res*, **58**: 808-814, 1998.
24. Sweeney, C. J., Miller, K. D., Sissons, S. E., Nozaki, S., Heilman, D. K., Shen, J., and Sledge, G. W., Jr. The antiangiogenic property of docetaxel is synergistic with a recombinant humanized monoclonal antibody against vascular endothelial growth factor or 2-methoxyestradiol but antagonized by endothelial growth factors. *Cancer Res*, **61**: 3369-3372., 2001.

25. Kim, I., Moon, S. O., Kim, S. H., Kim, H. J., Koh, Y. S., and Koh, G. Y. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem*, 276: 7614-7620, 2001.
26. Wajant, H. and Scheurich, P. Tumor necrosis factor receptor-associated factor (TRAF) 2 and its role in TNF signaling. *Int J Biochem Cell Biol*, 33: 19-32., 2001.

Table 1

	VEGF OD - SE (%Inhibition)	bFGF OD - SE (%Inhibition)	Plasma Parthenolide concentration 1 hr after oral gavage
Control	4.55 – 0.37 100%	3.79 – 0.25 100%	0 µM
Parthenolide 0.4 mg/kg	3.89 - 0.23 14% (<i>P</i> =0.29)	3.00 - 0.22 21% (<i>P</i> =0.07)	Undetectable
Parthenolide 4 mg/kg	4.01 - .27 12% (<i>P</i> =0.46)	2.26 – 0.24 41% (<i>P</i> =0.0002)	0.112 µM
Parthenolide 40 mg/kg	3.60 - 0.26 21% (<i>P</i> =0.09)	2.08 – 0.30 45.3% (<i>P</i> <0.0001)	0.169 µM

FIGURE LEGENDS:

Figure 1: (a) Electromobility Gel Shift Assay demonstrating NFkB-DNA binding of cultured HUVECs under various conditions. MNS: EBM-2 media with no supplements; HM: HUVEC media with supplements. Panels 1-3: Constitutive NFkB DNA binding with no supplements but increased with 50 ng/mL of bFGF and 100 ng/mL of VEGF. Panels 4-6: Less NFkB DNA binding in presence of supplements and further inhibition of binding by parthenolide (P). Panels 7-9 and 10-12 show increase NFkB DNA binding with bFGF and VEGF that is also inhibited by parthenolide.

(b) No change of STAT-3 or STAT-5 DNA binding in HUVECs in presence of parthenolide, bFGF or VEGF.

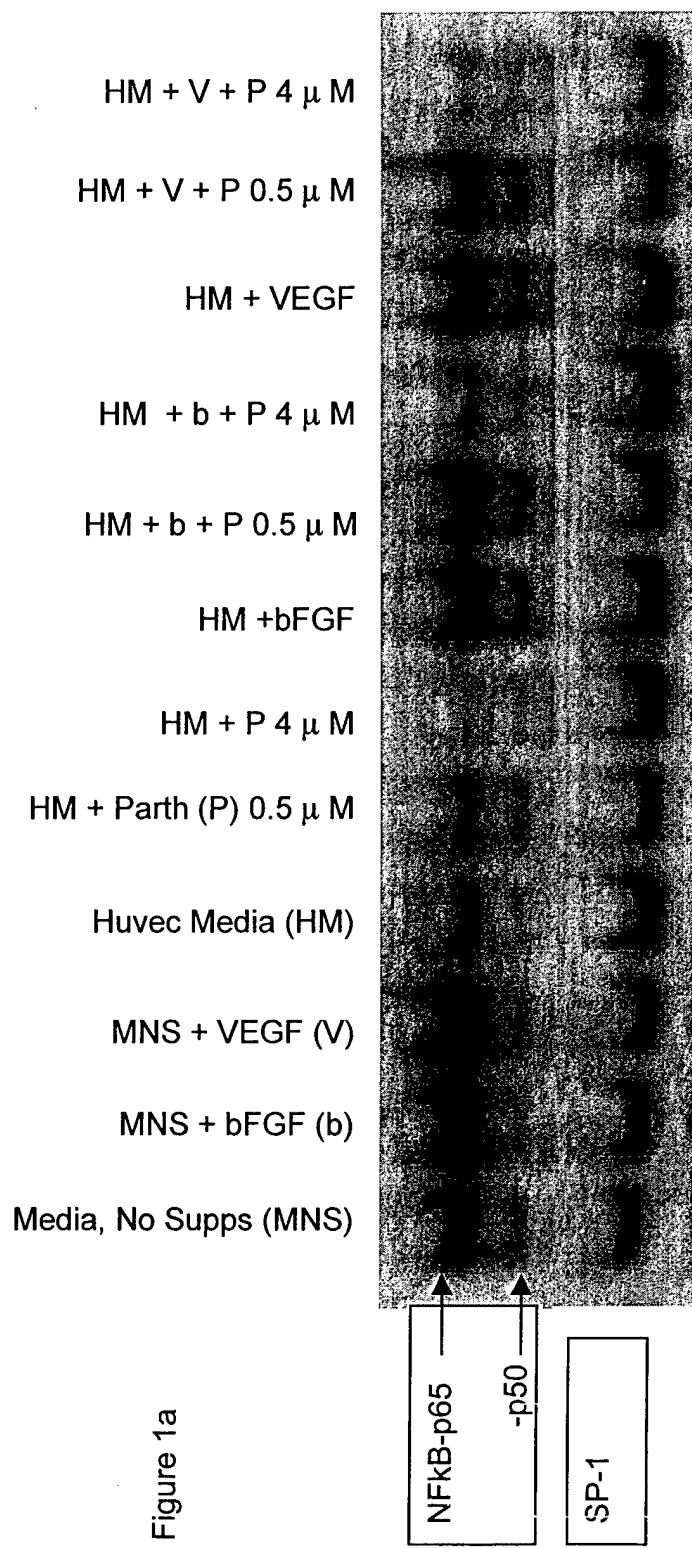
Figure 2: (a) Ribonuclease protection assay (RPA) of HUVECS exposed to VEGF, bFGF and parthenolide. Changes were only noted in TRAF-1: Present to a minor degree in cultured HUVECs but increased with bFGF and VEGF and decreased by parthenolide under all 3 conditions. (b) Southern blot of TRAF-1 after reverse transcriptase polymerase chain reaction confirming molecular effects found in (RPA).

Figure 3a: Inhibition of HUVEC proliferation by parthenolide. (Stimulation – 60 ng/mL of VEGF and 20 ng/mL of bFGF).

Figure 3b: Dose dependent inhibition of capillary formation by parthenolide.

Figure 3c: Dose dependent increase in apoptosis in HUVECs induced by parthenolide.

Table 1: Dose dependent inhibition of *in vivo* angiogenesis in a Matrigel plug assay by parthenolide as measured by optic density (OD) readings of hemoglobin concentration in each plug.



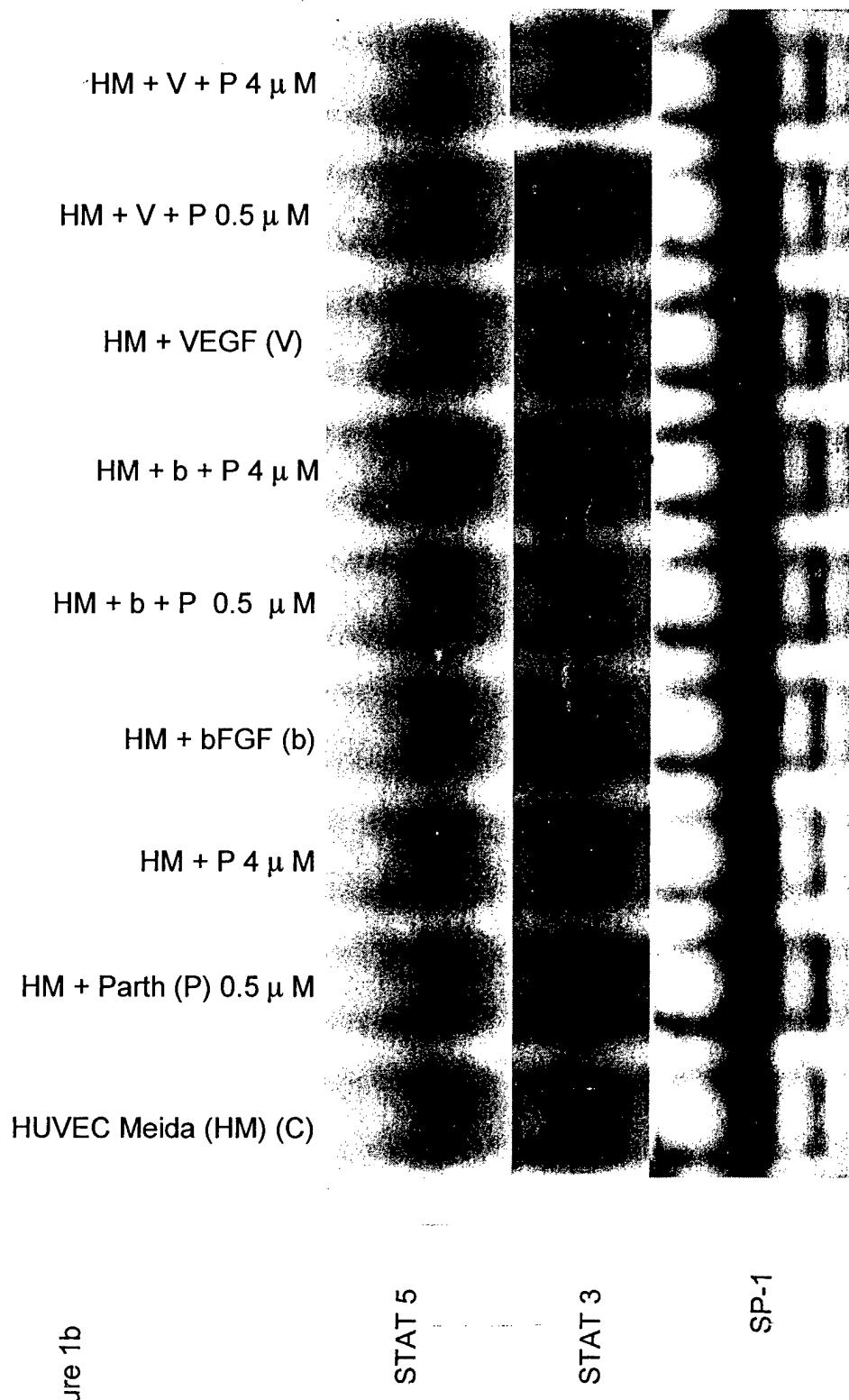


Figure 1b

Figure 2a

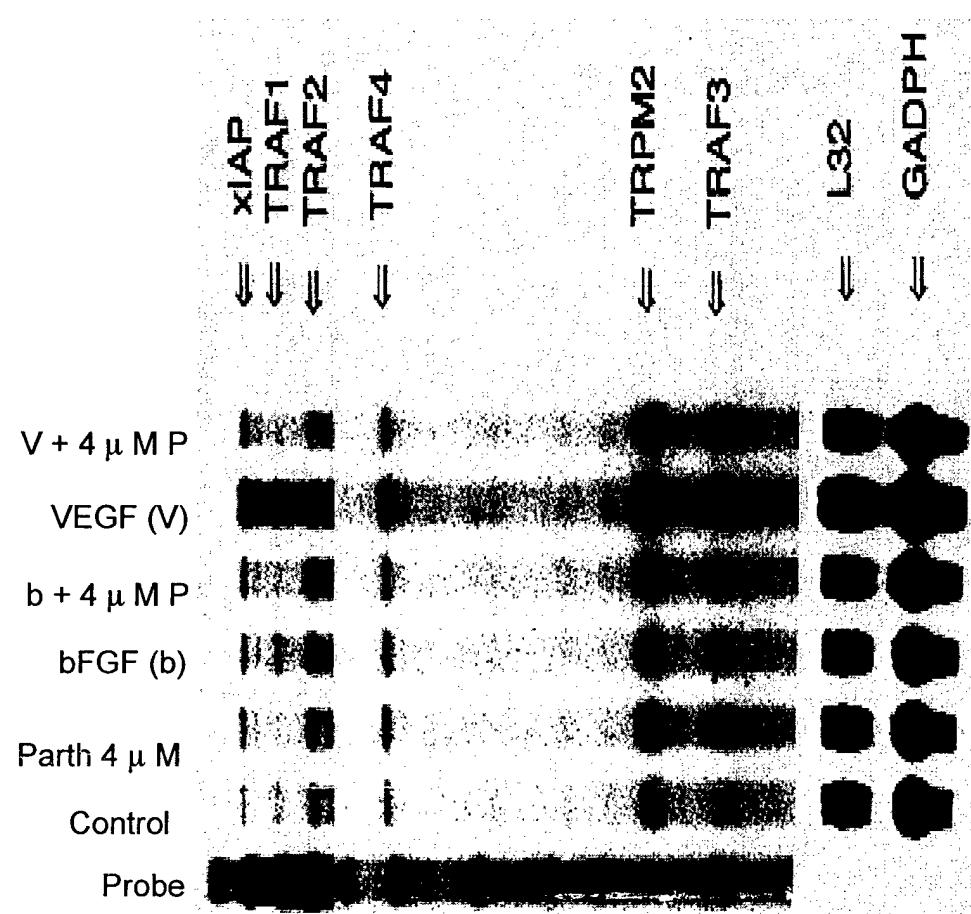


Figure 2b

